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14. ABSTRACT

We have recently demonstrated that a monoclonal antibody against prostate stem cell antigen (PSCA) can exert anti-tumor activity in a xenograft animal model, suggesting oncogenic activity of PSCA in prostate cancer. Therefore our goal is to elucidate the role of PSCA in the development of prostate cancer. A better understanding of PSCA function and its antibody activity will enable rational patient selection and trial design, all of which are particularly relevant to subsequent clinical trials of PSCA antibody. Better insight into the function of PSCA were obtained from the PSCA overexpressing experiments. We showed that PSCA promote significant cell growth as well as anchorage independent growth in prostate cancer cell line 22RV1, compared to that of normal cell line PZ-HPV7. Furthermore, PSCA appears to confer resistance to anoikis in both cell lines 22RV1 and LNCaP. These findings suggest that PSCA may enhance growth and survival of prostate tumor cells, particularly in the context of metastatic environment. This agrees with previous data that PSCA is upregulated in prostate cancer, especially in metastatic cancers. We have also generated the chimeric knockin mouse where human PSCA is expressed in place of mouse PSCA, and currently are expanding the colony.

15. SUBJECT TERMS
Prostate cancer, prostate stem cell antigen, preclinical mouse model, antibody therapy, antitumor activity

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INTRODUCTION

Previous studies from our laboratory have shown that PSCA is overexpressed in human primary and metastatic prostate cancers. Furthermore, we have demonstrated that a monoclonal antibody against PSCA can exert anti-tumor activity in a xenograft animal model, and that this activity is a direct effect mediated by cross-linking. These data suggest that PSCA may have growth-promoting and/or oncogenic activity in prostate cancer. Therefore our goal is to elucidate the role of PSCA in the development of prostate cancer in order to better understand the mechanism of action of PSCA antibody in tumor inhibition. Such insight will enable rational patient selection and trial design, all of which are particularly relevant to subsequent clinical trials of PSCA antibody. To accomplish this, we evaluate the requirement of PSCA expression for oncogenic activity in both xenografts and transgenic models of prostate cancer. We also generate a transgenic model of prostate cancer that express the human PSCA gene to determine the efficacy and toxicity of PSCA antibody therapy.

FINAL REPORT

Specific Aim 1. *Investigate the permissive role(s) of PSCA in cancer cell proliferation.*

Task 1. *Effect of gene silencing of PSCA in cell lines with endogenous PSCA expression.*

shRNA constructs against human PSCA, CSCG-GFP-siPSCA, with the most PSCA-knockdown efficiency were selected. The backbone of this lentiviral vector also contains GFP to monitor the infectivity and allow isolation of infected cells. Freshly isolated LAPC9 xenograft tumor cells were infected with the shRNA construct or CSCG-GFP alone and the effect on PSCA expression was examined by flow cytometry [figure 1]. After the initial infection, PSCA expression in the siPSCA group was reduced by ~ 43% (green line) compared to the CSCG-GFP infected cells (blue line).
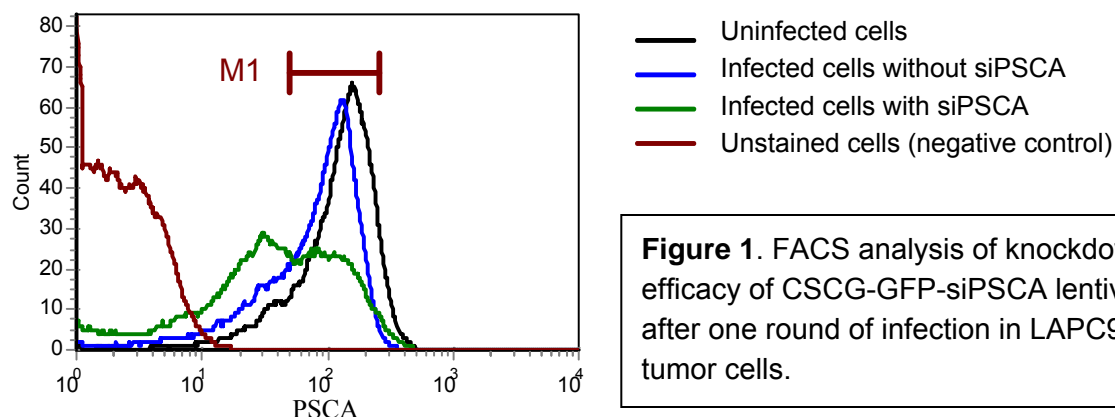

——	Uninfected cells
——	Infected cells without siPSCA
——	Infected cells with siPSCA
——	Unstained cells (negative control)

Figure 1. FACS analysis of knockdown efficacy of CSCG-GFP-siPSCA lentivirus after one round of infection in LAPC9 tumor cells.

While PSCA was not suppressed completely, with repeated rounds of lentiviral infection we expected to obtain high percentage of reduction in PSCA expression. However, LAPC9 cells are xenografts which cannot be stably maintained in vitro as other cell lines, thus it was a challenge to perform repeated infections while keeping these cells alive in vitro. To overcome this problem, we tested and established

conditions for primary culturing of LAPC9 for 5 to 10 days ex vivo [figure 2]. This time frame allows us to repeat infection of the cells, check and select for GFP positive cells.
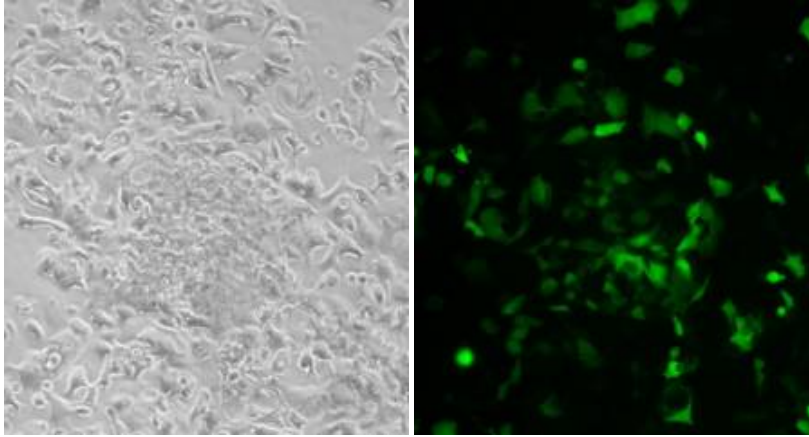

Figure 2. Ex vivo primary culture of LAPC9 cells infected with CSCG-GFP-siPSCA lentivirus. Left panel: light field; right panel: dark field.

We proceeded to in vivo experiment, whereby freshly isolated LAPC9 cells were infected with CSCG-GFP-siPSCA or CSCG-GFP alone, and sorted for GFP positive cells after one week. Both control and siPSCA cells were innoculated back into mice and the tumor growth monitored over time. There were no apparent difference in tumor growth or size between both groups. When the tumors were harvested and analyzed for PSCA by FACS, the percentage of PSCA positive cells and the signal intensity in the siPSCA group was comparable to that of control group. This suggests that while the cells used for innoculation was 100% GFP positive, knockdown of PSCA was not 100% effective.

Task 2. *Effect of overexpressing PSCA in normal prostate epithelial cells (PrECs) and other heterologous cell lines.*

PSCA (or the control dsRed, red fluorescent protein) was overexpressed by lentiviral transduction into the normal prostate cell line PZ-HPV7, or the prostate cancer cell line 22RV1. All sublines were sorted by flow cytometry to obtain PSCA+ cells, and compared in terms of growth at different densities by MTT assay. For the normal PZ-HPV7 cell line, PSCA overexpression appeared to have no significant growth advantage over control cells at either low or high density. In contrast, in the prostate cancer cell line 22RV1, PSCA overexpressing cells showed markedly higher growth rate compared to dsRed control at lower density [figure 3], while at higher density the effect was less prominant. Thus PSCA may promote cell growth in cancer cells while it has no such effect in normal cells.

Anchorage independent growth was also assessed. PSCA-overexpressing or control dsRed cells were seeded at clonal density as single cells in soft agar and maintained for three weeks. PSCA overexpression promoted significantly higher anchorage independent growth of 22RV1 on soft agar over dsRed control, while the effect was less prominant in the normal cell line PZ-HPV7 [figure 4]. Of note, we also tested LNCaP and LNCaP-PSCA cells, where there was a slight growth increase in PSCA expressing cells but no difference in anchorage independent growth.

Cell adhesion to extracellular matrix could induce signal transduction cascades within the cell, affecting cell growth, differentiation and death [3]. When cell adhesion is disrupted, cells undergo apoptosis, a phenomenon described as anoikis [4]. It has been suggested that tumor cells are able to acquire anoikis

resistant capability, which may implicate their metastatic potentials [5, 6]. Recently, GPI-anchored molecules have been shown to be able to mediate anoikis [7, 8]. As such, we explored the possible function of PSCA in relation to cell adhesion and anoikis, as a GPI-linked cell surface molecule.

22RV1 and 22RV1-PSCA cells were grown in polyHema coated tissue culture dish to prevent adhesion for 1 – 7 days. At each time point cells were harvested and stained with Annexin V to detect apoptosis by flow cytometry. We also included a second subline of 22RV1-PSCA which was independently established. After 2 days in non-adherent condition, there was a twofold increase in anoikis in parental cells compared to PSCA overexpressing cells [figure 5]. This effect was more prominent at 7 days in non-adherent culture. Similar results were obtained in LNCaP and LNCaP-PSCA cells. This suggests that PSCA may promote resistance to anoikis in prostate cancer cells.
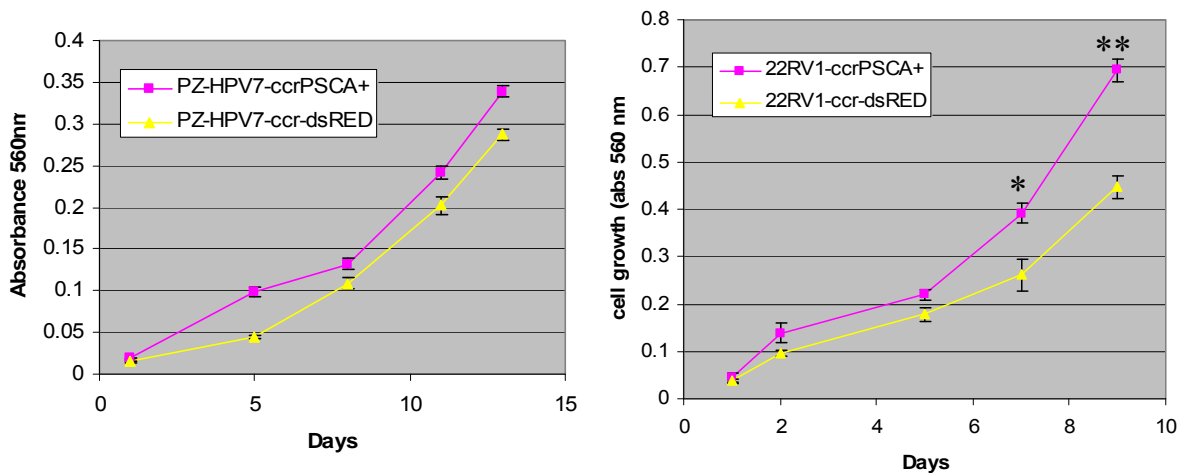

Figure 3. Effects of overexpressing PSCA on cell growth in normal prostate cell line PZ-HPV7, and prostate cancer cells 22RV1. *P < 0.011; **P < 0.005.
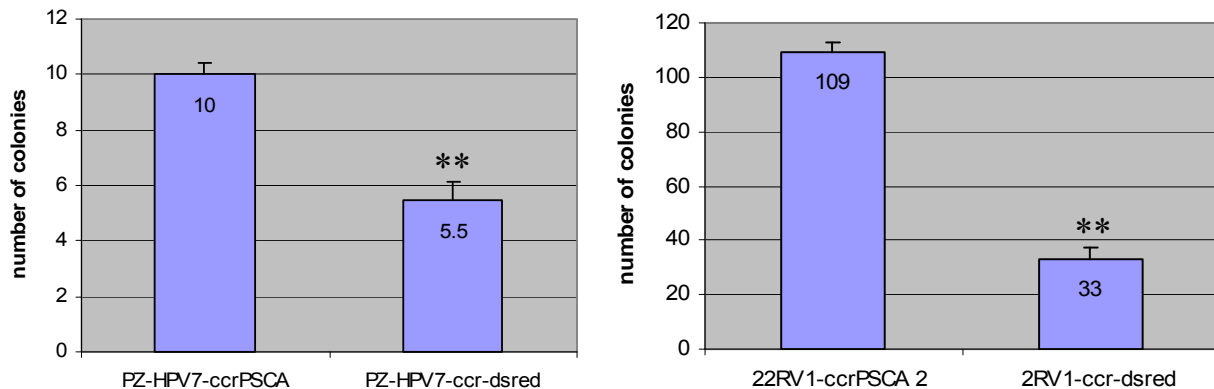

Figure 4. Effects of PSCA on anchorage independent growth in normal prostate cell line PZ-HPV7. and 22RV1 prostate cancer cells. **P < 0.005. Colonies > 300 μm were counted.
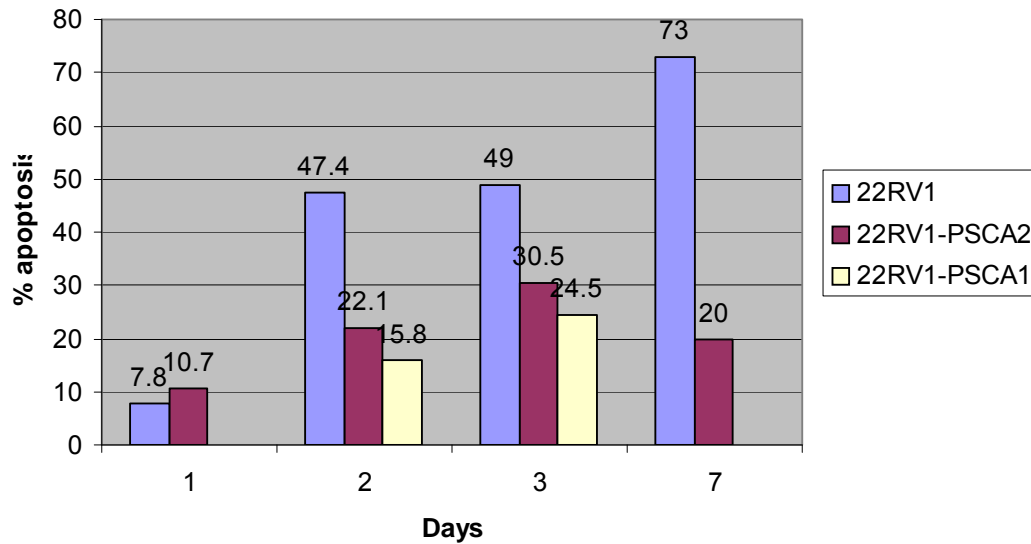
Figure 5. Effects of PSCA on anoikis in 22RV1 and 22RV1-PSCA grown in non-adherent condition. Anoikis was measured by percentage of Annexin V staining.

Specific Aim 2. *Evaluate the role of PSCA in the causation and progression of prostate cancer*.

Task 1. *Effect of PSCA deficiency in the PTEN$^{-/-}$ transgenic model*.

Due to the difficulty in obtaining the PSCA$^{-/-}$/conditional PTEN$^{-/-}$ compound mice, we were planning to try an alternative approach. This involves obtaining prostate cells from the PSCA knockout mice and infecting the cells with a PTEN knockdown construct, following by combination with the mouse embryonic urogenital sinus mesenchymal cells and engraftment under the kidney capsule of immunodeficient host mice. Such assay has been utilized previously [1] to study murine prostate tissue regeneration, and more recently [2] as a tool to evaluate the result of genetic perturbation in murine prostate cells within 8 weeks. However we encountered difficulties in getting enough mouse prostate cells for infection and for subsequent tissue recombination experiment.

Task 2. *Effect of PSCA deficiency in the Nkx3.1$^{-/-}$ transgenic model*.

We examined the PSCA$^{-/-}$/Nkx3.1$^{-/-}$ double homozygous compound mice and their Nkx3.1$^{-/-}$ control cohort for difference in the duration to PIN formation in the prostate. The first problem we encountered was that all the mice took much longer time to develop prostatic intra-epithelial neoplasia (PIN), even in the Nkx3.1$^{-/-}$ control group. The most obvious abnormalities were observed in the anterior prostates at 16 weeks, and here there was no difference between the double knockout group and the control group. Following this time point, we acquired tissues from mice groups at 32 weeks and one year for histology assessment, and found no clear difference between the double homozygous compound mice and their Nkx3.1-/- control group. Therefore in this model, PSCA deficiency did not accelerate or delay PIN formation.

Specific Aim 3. *Assess the efficacy and physiological effects of the antibody in a preclinical model expressing human PSCA.*

Task 1. *Development of transgenic model of prostate cancer expressing human PSCA.*
We revised our strategies for developing this preclinical model. Initially we proposed to generate a transgenic mouse overexpressing human (h)PSCA using the human PSCA promoter while its mouse counterpart is not disturbed, and subsequently cross it with PTEN null mice. However, we did acknowledge the concern that targeting hPSCA alone may not be sufficient to inhibit tumor growth since the expression of mouse PSCA is not targeted. Therefore, our alternative approach was to specifically place the hPSCA cDNA under the mouse promoter by "knock-in" gene targeting, thus effectively silencing the mouse PSCA by expressing its human counterpart.

We screened the CITB mouse BAC library by oligonucleotide probe, and three clones were identified. Upon further confirmation by PCR using primers specific for mouse PSCA, only one BAC clone was positive. A EcoRI/XhoI 9.8 kb fragment containing mPSCA gene was isolated and subcloned into Bluescript plasmid. From here, a 5' and 3' genomic arms were generated by restriction digest and PCR cloning. The 5' arm was 3 kb, starting from the transcriptional start site going upstream, while the 3' arm started within the first intron of mPSCA and ended at XhoI site at the 3' end of the gene. Thus the first exon and first half of the intron in mPSCA gene was targeted to be deleted and replaced with hPSCA cDNA. The genomic arms were strategically subcloned into a gene targeting vector, pGKneo-F2L2DTA [figure 6].
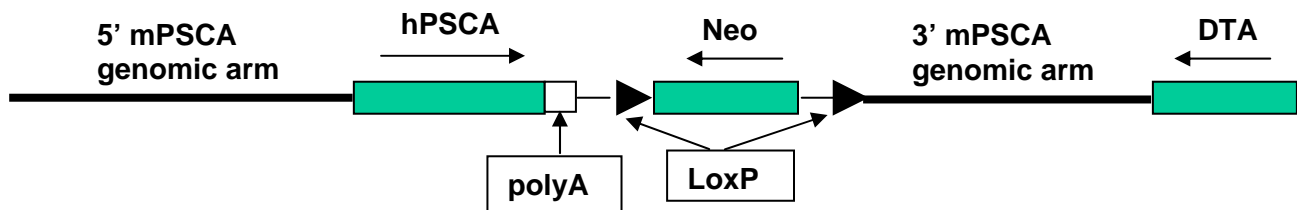

Figure 6. Schematic representation of gene targeting construct of hPSCA. Neo – neomycin resistance, for selection of recombinant ES clone; DTA – diptheria toxin A, selecting against random integration; LoxP – site for Cre recombinase to remove neomycin cassette.

The sequence of this vector was confirmed, and the linearized vector was introduced into 129sv ES cells. 288 neomycin resistant positive clones were picked, expanded to harvest genomic DNA, and the ES DNA samples were screened by PCR to identify recombinant clones. Out of 96 samples tested, 45 samples were positive for recombination. 12 positive and 1 negative samples were further analyzed by Southern hybridization, and confirmed to have the correct recombination. Three confirmed ES clones were picked and submitted for injection into C57Bl/6 blastocysts. The resultant founders, consisted of five males and three females, were genotyped by PCR and 4 males were positive (2 strong and 2 weak). We are in the process of breeding these male founders to see which one could stably pass on the knockin hPSCA gene.

Task 2. *Studies of PSCA antibodies in transgenic models*.

This task will be performed once the preclinical model is established. At this point, it is beyond the duration of this grant.

KEY RESEARCH ACCOMPLISHMENTS

Year 1
- Establish conditions for infecting LAPC9 xenograft tumor cells in vitro.
- Confirm knockdown ability of siPSCA lentivirus.
- Examine PSCA$^{-/-}$/Nkx3.1$^{-/-}$ double knockout mice at early time point (16 weeks) and find no difference in the extent of time taken to PIN formation compared to control group.
- Revise approach to breeding PSCA$^{-/-}$/conditional PTEN$^{-/-}$ compound mice by using the tissue recombination assay.
- Revise approach to making the preclinical model by using gene targeting "knock-in" technology.

Year 2
- In vivo experiment of LAPC9 xenograft tumor cells.
- Overexpression of PSCA promotes cell growth in vitro in cancer cell line 22RV1, but not normal cell line PZ-HPV7. However, anchorage independent growth were enhanced by PSCA in both cell lines.
- Examine PSCA$^{-/-}$/Nkx3.1$^{-/-}$ double knockout mice at later time points (32 weeks and one year) and find no difference in the extent of time taken to PIN formation compared to control group.
- Generation of the human PSCA gene targeting construct for preclinical mouse model.

Year 3
- Overexpression of PSCA promotes resistance to anoikis in vitro in LNCaP and 22RV1 cells.
- Confirm correct sequence of the gene targeting construct and introduce the construct into ES cells.
- Identify correct recombinant ES clones by PCR and Southern hybridization.
- Generate chimeric hPSCA knockin founders.

REPORTABLE OUTCOMES

- Generation of human PSCA overexpressing cell lines: 1/ 22RV1-PSCA, 2/ PZ-HPV7-PSCA. These lines are being used to test the affinity of PSCA-derived antibodies.
- Development of animal model: the human PSCA knockin mouse. This mouse model is being used to test the sensitivity of the PSCA-derived imaging agents.

CONCLUSION

The requirement of PSCA expression for oncogenic activity in both xenografts and transgenic models of prostate cancer were investigated in aim 1 and aim 2. We encountered difficulties in using the LAPC9 xenograft cells to study the effect of suppressing PSCA, despite having worked out conditions to improve lentivirus infection effeciency. The in vivo experiment to study the effect of knocking down PSCA in LAPC9 tumor cells were performed, however the results showed no difference in tumor growth, and assessment of PSCA expression showed no difference between control and knockdown tumor. This result underscores the problem of achieving long term efficient suppression of PSCA in this model, and the outcome remains inconclusive. On the other hand, better insight into the function of PSCA were obtained from the PSCA overexpressing experiments. We showed that PSCA promote significant cell growth as well as anchorage independent growth in prostate cancer cell line 22RV1, compared to that of normal cell line PZ-HPV7. Furthermore, PSCA appears to confer resistance to anoikis in both cell lines 22RV1 and LNCaP. These findings suggest that PSCA may enhance growth and survival of prostate tumor cells,

particularly in the context of metastatic environment. This agree with previous data that PSCA is upregulated in prostate cancer, especially in metastatic cancers.

In aim 2, we generated the PSCA$^{-/-}$/Nkx3.1$^{-/-}$ double knockout mice to study the requirement of PSCA in early initiation of prostate cancer. Throughout all three time points (16, 32, 52 weeks), there was no signficant difference in the time duration to PIN formation between the double knockout mice and the control group (Nkx3.1 knockout). Therefore in this mouse model, PSCA deficiency did not accelerate or delay PIN formation. However, this does not rule out the role of PSCA in prostate cancer. It is possible that PSCA is more important in the progress to metastatic cancer than in the primary tumor stage, since we have shown that PSCA protein and mRNA are highly elevated in clinical specimen of prostate cancer metastases [9].

In aim 3, we successfully engineered and generated a human PSCA knockin mouse model that express human PSCA in place of mouse PSCA. The chimeric founders were genotyped and are being bred to determine which line is stable past the F1 generation. Ultimately, this model will be very useful to determine the efficacy and toxicity of PSCA antibody therapy by crossing with other model of prostate cancer, such as the conditional PTEN knockout.

Overall we have gained important information in elucidating the role of PSCA in the development of prostate cancer. We have shown that PSCA overexpression promotes prostate cancer cell growth as well as resistance to anoikis in vitro. In parallel, we showed that absence of PSCA did not affect prostate cancer initiation in the Nkx knockout mouse model. These results suggest that PSCA may be important for growth and survival of prostate cancer cells, but not in the cancer initiation stage. The role of PSCA in prostate cancer metastasis remains unknown. Based on these results, we do not plan to pursue the knockdown experiment in LAPC9, and the crossing of PSCA knockout to the PTEN conditional knockout. On the other hand, with the successful development of the human PSCA knockin mouse model, we have proceeded to evaluate the expression of hPSCA in various tissues, and are planning to use this model to test the efficacy of a PSCA-derived antibody. This would involve crossing this knockin line to a mouse model with metastatic potential (eg. TRAMP mice), and use the compound mouse model as a preclinical platform to test drug combination.

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